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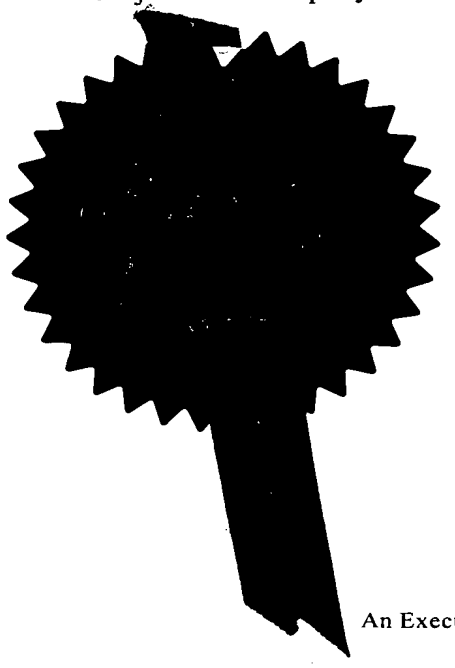

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	Full name, address and postcode of the applicant	University of Leeds **LEEDS** **LS2 9JT**
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6	Priority applications	Country	Priority App No	Date of Filing

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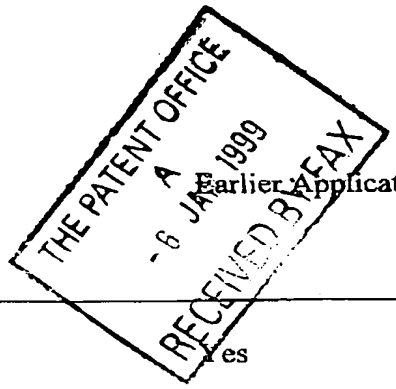

7	Parent application (eg Divisional)	Earlier Application No	Date of Filing
8	Statement of Inventorship Needed?	Yes	
9	Number of sheets for any of the following (not counting copies of same document)		
	Continuation sheets of this form		
	Description	19	
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	Abstract		
	Drawings	6	
10	Number of other documents attached		
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	P10/77		
	Other documents		
11	I/We request the grant of a patent on the basis of this application.		
	Signature Lisa Brown		6 January 1999
12	Name and daytime telephone number of person to contact in the United Kingdom	Dr Lisa Brown 0113 225 8350	

Wound and Tissue Healing and Orofacial Clefting

The present invention relates to the isolation of a nucleic acid molecule and the protein encoded thereby; and the use of these products as therapeutic agents particularly, but not exclusively, in gene therapy and/or tissue repair such as, without limitation enhancing wound and tissue healing and for the treatment of orofacial clefting.

Orofacial clefting is the most common human malformation, with an overall incidence of approximately 1 in 600 births. Cleft palate (CP) requires complex management and follow up by multi-disciplinary medical and surgical teams. It has a major impact on both communication and psychological morbidity. Both animal and human studies have shown that cleft palate can be the end result of a number of different aetiological processes. Amongst the teratogenic agents that can cause CP are common drugs (diazepam, sodium valproate, alcohol etc.). Maternal diabetes also confers an increased risk of CP. However, twin studies and familial segregation analyses in a number of different populations have consistently shown that there is a major genetic component to the aetiology of this common developmental abnormality. Despite this, little is known about the specific genetic defects underlying CP. The care of children with clefting is now concentrated in supra-regional centres where the necessary resources can be assembled.

Isolated cleft palate (CPO) is a common human malformation, with a total birth incidence of 1 in 1250 in the West of Scotland [FitzPatrick et al. 1994]. Significant familial clustering [Carter et al. 1982; Shields et al., 1981; Christensen & Fogh-Andersen 1993] and twin studies [Shields et al. 1979] have both suggested that there is a major genetic component in the etiology of CPO. These studies and others [Fogh-Andersen, 1942] have also shown that CPO and cleft lip with or without cleft palate (CL(P)) are genetically distinct subgroups of orofacial clefting. CPO is a common feature of chromosomal abnormalities, affecting ~15% of all cases of simple autosomal aneuploidy (Schinzel 1994) and is associated with more than 370

different malformation syndromes (Baraitser & Winter, 1997). However, in non-syndromic CPO, relative risk ratio analyses have indicated that there may be a relatively small number of interacting causative loci [FitzPatrick & Farrell, 1993; Christiensson & Mitchell, 1996]. As yet, no disease-causing mutations in non-syndromic CPO have been identified. Here we report clinical, cytogenetic, molecular and statistical evidence for the existence of a previously unrecognised locus for CPO located at 2q32.

Development of the secondary palate, particularly in mice, has been used as a paradigm in developmental biology, and extensive descriptive studies of the distribution of proteins with putative roles in this process have been published. This knowledge base has led to the extensive use of candidate gene approaches in attempts to unravel the genetic basis of human cleft palate. However, these studies have met with limited success. Purely genetic approaches, in contrast, by the identification of genes causing rare syndromic forms of cleft palate, have yielded greater insights. An example of this is the positional cloning of the gene responsible for Treacher Collins syndrome. Experimental mouse models are another source of valuable information, for example the transgenic knock outs of TGFβ3 and MSX1. However, extrapolation from these models by testing for genetic association in human populations has been generally unsuccessful.

There are a number of semi-dominant and recessive mouse models of isolated cleft palate, for example Twirler (Tw) and Dancer (Dc). These provide excellent opportunities to identify potential susceptibility loci for human CP via the positional cloning of the mouse genes. However, none of the loci for these mouse models lies within a region of conserved synteny with the human chromosome 2q32 region which is the subject of this application.

We have recently addressed the problem of identifying susceptibility loci for human malformations by utilising the common phenomenon of autosomal aneuploidy. Using the Human Cytogenetics Database, we have statistically analysed the entire set

of information available on post-natally ascertained cases of simple autosomal aneuploidy in a manner not previously attempted [Brewer et al, 1998]. This has allowed the identification of specific autosomal regions that are significantly associated with particular malformations. 37 different malformations were studied, covering a variety of different developmental processes. For cleft palate, 5 different putative loci were identified. The validity of the approach was confirmed by the identification of loci on 4p and 4q, which have previously been suggested to harbour CP susceptibility genes as a result of association or linkage studies. Unexpectedly a major new locus has been identified at 2q32-q33. Interestingly, the penetrance of haploinsufficiency in causing cleft palate at this locus appears to be higher than that of the other four loci identified in our study. This region has not previously been implicated in the pathogenesis of CP. We have therefore gone on to obtain further independent evidence for the importance of this locus.

We have now identified a human gene which, when dysfunctional, results in cleft palate in man. Replacement of gene function is therefore useful in the treatment of cleft palate and the related cleft lip. There is precedent that disruption of the TGFβ3 gene, for example, leads to the development of cleft palate in animal models. There is also precedent that the use of TGFβ3, for example, is beneficial in the treatment of wounds to achieve enhanced rates of healing. The gene and the protein of the present invention are also therefore useful therapeutically to enhance wound healing. They are also useful clinically to improve tissue repair in other clinical contexts, for example in inducing repair of damage to cartilage or boney tissue.

We have thus successfully tested the novel hypothesis that there is a major locus for cleft palate located at human chromosome 2q32. We present the unexpected results of an extensive study which points to the existence of such a gene. We have now cloned and characterised this cleft palate gene using unique clinical material.

Over the last three years, it has been our practice to perform karyotype analysis on all children with cleft palate. Recently, we have ascertained an 11 year old girl with a de

novo balanced reciprocal translocation between chromosomes 2q32 and 11p14. This patient has a midline posterior cleft of the soft palate, mild learning difficulties and subtle craniofacial dysmorphism. We then studied a second patient with a similar clinical phenotype and another de novo balanced reciprocal translocation, this time between chromosomes 2q32 and 7p21. These patients have remarkably consistent clinical features. The existence of 2 different individuals with balanced translocations, possibly involving a common break point at 2q32, strongly supported the conclusion of our aneuploidy study [Brewer et al, 1998], that an important susceptibility locus for cleft palate exists in this cytogenetic region.

We have therefore taken a molecular cytogenetic route to further analyse these two patients. Initially, we wished to establish that the two break points on 2q32 were indeed located in the same region. A number of experiments using single chromosome painting were performed, which confirmed that both re-arrangements were apparently simple balanced reciprocal translocations and that to a first approximation, they occurred at the same place on chromosome 2q (Figure 1). Next, we tested the possible involvement of a number of potential candidate genes in this region. Yeast artifical chromosomes (YACs) containing these candidates were isolated and each was used for fluorescent in situ hybridization (FISH) analysis. This showed, for example, that the fibronectin and IGF binding protein 5 (IGFBP5) genes are both telomeric to the breakpoints (Figure 2).

We then isolated a total of 70 YACs using genetic markers distributed across the whole 2q32-q33 region. FISH analysis was performed systematically using each of these YACs, in order to position the breakpoints more precisely. Typical results from this study are presented in Figure 3. In support of our initial hypothesis, the FISH analyses confirm that the 2q breakpoints in both patients do indeed lie in the same small interval. We narrowed this region to less than 2 centimorgans, by virtue of the fact that YACs containing the marker D2S311 are centromeric to both patients' breakpoints, while YACs containing D2S309 are telomeric to the breakpoints, again in both patients (Figure 3 shows Case 1 for example).

On the basis of this information, a YAC contig spanning this interval has been constructed. This has enabled us to resolve a number of uncertainties in current genetic maps of this region. We have placed 23 genetic markers within this small interval and isolated a total of 33 corresponding genomic clones. Our FISH results initially narrowed the region containing both breakpoints to <600 kb. The YAC clone 854H8 (Fig. 4, 5) crosses both breakpoints. Clearly, this means that we have cloned the novel CP gene. The novel gene is defined by the break points on chromosome 2q32 in patients "Case 1" (0213) and "Case 2" (0145) at the point where they occur within YAC 854H8, which has been deposited with the National Patent Collection of Microorganisms, Aberdeen, Scotland. Cell lines from Case 1 are available in the form of lymphoblastoid cells from ECACC (Porton Down, UK).

The YAC clone spanning both breakpoints was used to screen flow-sorted, chromosome 2 specific cosmid and PAC libraries. A PAC and cosmid contig of the breakpoint region was constructed by a combination of fingerprinting and sequencing. Individual clones were used in further FISH analysis. This approach leads us to the PAC "CP-1" which has also been deposited as above, which defines the region harbouring the CP gene. We then took the most direct route to isolating the gene itself, which was sequencing of this PAC clone.

The resulting genomic DNA sequence was used for gene identification by a combination of EST database searching and computational prediction. We screened cDNA libraries to isolate clones corresponding to transcripts in this region (Fig. 6). Together with the genomic sequence, these clones provided us directly with information about the intron-exon structure of the gene, and its promoter. In parallel studies, we also isolated the corresponding mouse gene by techniques well known in the art, which will be a prerequisite for transgenic studies.

Availability of the CP gene now allows us to analyse our material from patients with cleft palate. We can look for microdeletions at this locus, using FISH analysis of

patients with apparently normal karyotypes. We also undertake sequencing of the gene, using cDNA or genomic DNA substrates as appropriate, in these patients. These diagnostic tests are useful clinically in the management of patients with cleft palate and in genetic counselling of them and their families.

In view of evidence implicating a number of known biologically active molecules, both endogenous and exogenous, in the aetiology of CP, it is interesting to examine the influence of such agents on the expression of the newly isolated 2q32 CP gene. This involves the construction of reporter plasmids to examine CP gene promoter function. Such studies can then be followed up by a more detailed functional analysis of the promoter sequences including deletion mapping and direct examination of the transcriptional response of the gene to a variety of agents, by methods well known in the art. Thus the subject of the present invention is of value to the pharmaceutical industry for the toxicological evaluation of potential new drugs. Analysis of their effects on expression of the CP gene which is the subject of the present invention, will enable prediction of their possible teratogenicity.

As discussed above, we isolated mouse genomic clones from libraries of strain 129 DNA isogenic with the ES cells in use in our laboratories. Having confirmed the organisation of the mouse gene, we performed transgenic knockout experiments using standard approaches. One specific approach involved the use of the IRES-βgeo targetting construct, which as well as eliminating function of the CP gene, allows monitoring of its expression pattern in the embryo by simple staining for -βgalactosidase rather than extensive use of in situ hybridization. Such a transgenic approach also facilitated analysis of the effects of recognised teratogens on the expression of this gene in vivo.

Availability of a transgenic model enables us to undertake other interesting studies. For example, as the mutation in mouse is not completely penetrant, we are in a position to utilise mouse genetic approaches to map modifier loci. These are likely to have homologues in man which could well be of clinical significance.

In conclusion, we believe we have demonstrated the unexpected existence of an important locus on human chromosome 2q32 causing cleft palate. We have cloned this CP gene and found mutations in a large cohort of patients with cleft palate. Given the problems of day-to-day clinical management of patients with this distressing condition, we also expect that the diagnostic tools described herein will be rapidly exploited clinically and possibly suggest approaches to prevention strategies for this common malformation.

It is therefore an object of the present invention to provide a tissue repair protein for use as a therapeutic agent.

It is a further object of the present invention to provide a tissue repair protein for use as a diagnostic agent.

According to the first aspect of the invention there is therefore provided a cloned nucleic acid contained in the Yeast Artificial Chromosome species 854H8.

According to a further aspect of the invention there is provided an isolated nucleic acid molecule molecule from the 2q32 chromosome encoding a tissue repair protein wherein said isolated nucleic acid molecule has a nucleotide sequence which hybridises to a nucleic acid under high stringency conditions.

Preferably, hybridisation occurs under stringent conditions such as 1 x SSC, 0.1% SDS at 65 °C.

According to a further aspect of the invention there is provided an isolated polypeptide encoded by the nucleic acid molecule according to the invention.

According to a yet further aspect of the invention there is provided a delivery vehicle comprising the isolated nucleic acid molecule of the invention.

Reference herein to the term delivery vehicle is intended to include any vector whether a viral vector or otherwise for example, without limitation, an adenovirus, a retrovirus, a herpesvirus, a plasmid, a phage, a phagemid or a liposome.

Ideally said delivery vehicle is adapted for administration, for example, but without limitation, by suitable formulation into a suspension.

More preferably, said delivery vehicle is adapted to deliver said nucleic acid molecule to selected tissue. Thus the delivery vehicle is provided with means to enable the nucleic acid molecule to be targeted to a specific site. The nature of the means comprises conventional technologies well known to those skilled in the art for example, without limitation, in the instance where the delivery vehicle is a viral vector said viral vector is provided with surface protein adapted to ensure the viral vector binds to and/or penetrates specific target tissues. Thus, in this way, the nucleic acid molecule of the invention can be used in gene therapy treatments.

According to a yet further aspect of the invention there is provided antibodies raised against the polypeptide of the invention. Ideally the antibodies are monoclonal and more ideally genetically engineered to be humanised.

It will be apparent to those skilled in the art that the antibodies of the invention can be used to determine the expression of the polypeptide of the invention in selected target tissue.

According to a further aspect of the invention there is provided a method for the treatment of tissue repair of chondrogenic/cartilage/bone/ossified tissue comprising administering to a patient suffering from orofacial clefting or other skeletal disease or injury the nucleic acid molecule and/or polypeptide of the invention.

According to a further aspect of the invention there is provided a method for the treatment of wounds comprising administering to a patient suffering from a wound the nucleic acid molecule and/or polypeptide of the invention.

According to a further aspect of the invention there is provided a method of tissue repair of cartilage or boney tissue comprising administering to a patient suffering from orofacial clefting or other skeletal diseases or injury the nucleic acid molecule and/or polypeptide of the invention.

In this preferred embodiment of the invention said nucleic acid molecule is administered by the incorporation of said nucleic acid molecule into a delivery vehicle as herein described and ideally the method of treatment involves the use of gene therapy.

Embodiments of the invention will now be described by way of example only with reference to the following figures wherein:

Figures 1 A and 1 B represent chromosome painting of chromosome 2q in the patients "Case 1" and "Case 2" respectively,

Figures 2 A and B represent fluorescent in situ hybridisation of IGF binding protein 5 in patients Case 1 and Case 2 respectively,

Figures 3 A and B represent fluorescent in situ hybridisation of YAC's containing the marker D2S311 or D2S309 respectively in patient Case 1. A similar picture is obtained for Case 2 (data not shown).

Figure 4 represents fluorescent in situ hybridisation of YAC 854H8 in patient Case 1,

Figure 5 represents fluorescent in situ hybridisation of YAC 854H8 in patient Case 2,

Table 1 represents oligonucleotides used in the study,

Table 2 represents the genetic map of the CP-1 region.

We report two unrelated individuals with strikingly similar clinical features, in whom there are apparently balanced de novo cytogenetic rearrangements involving the same region of chromosome 2q. Both children have cleft palate, facial dysmorphism and mild learning disability. Their karyotypes were originally reported as 46,XX,t(2;7)(q33;p21) and 46,XX,t(2;11)(q33;p14). However, we now describe molecular cytogenetic analyses that have localised the translocation breakpoint in both cases to a region of some 0.3 Mb between markers D2S311 and D2S309. This suggests that the true location of these breakpoints is 2q32 rather than 2q33. To obtain independent support for the existence of a novel locus for cleft palate on 2q32, a detailed statistical analysis was performed of all cases in the Human Cytogenetics Database of non-mosaic single contiguous autosomal deletions associated with orofacial clefting. This revealed 2q32 to be one of only three chromosomal regions in which haploinsufficiency is highly significantly associated with isolated cleft palate. In combination, our data provide strong evidence for the existence at 2q32 of a gene that is critical to the development of the secondary palate. The close proximity of the two chromosomal breakpoints also makes the positional cloning of this gene a realistic possibility.

SUBJECTS AND METHODS

Case 1

Case 1, the fourth child of healthy, non-consanguineous parents, was delivered at 38 weeks' gestation, weighing 2.95kg. Cleft palate was noted at birth. Delayed motor development was apparent at four months and the patient did not walk until two years. Particular problems were noted with the acquisition of language skills. She underwent repair of her cleft palate at eighteen months of age and required

pharyngoplasty at eleven years. Her hearing is normal. She has a prominent nasal bridge, a small mouth and long, slender fingers. Her growth has been satisfactory, and her height has always been on or above the 50th centile. Her weight was below the 10th centile until the age of five but at the age of ten is on the 75th centile. Her head circumference is on the 50th centile. She has moderate learning disability. Blood chromosome analysis revealed an apparently balanced reciprocal translocation with the karyotype 46XX, t(2;7)(q33;p21). Parental karyotypes were normal. FISH analysis showed no deletion of 22q11.22.

Case 2

Case 2 is a female delivered at term after an uneventful pregnancy. At birth she was noted to have cleft palate and minor facial dysmorphism. In addition to repair of her palate, she has required surgical correction of a convergent squint. On examination at the age of 8 years, she has fair hair and skin, a long, narrow face with apparent hypotelorism, a prominent nasal bridge and pinched appearance above the nares, and a small mouth and jaw. She has abnormal dermatoglyphics with a reduced ridge count. She is of slender build, with height between the 75th and 90th centiles and weight on the 10th centile. She has mild global developmental delay, particularly in language skills, and is one year behind her peers in a mainstream school. Chromosome analysis revealed an apparently balanced reciprocal translocation with the karyotype 46,XX,t(2;11)(q33;p14). Parental karyotypes were normal. FISH analysis showed no deletion of 22q11.22.

Molecular cytogenetic analysis

Our initial working map across the 2q breakpoint region (Dib et al. 1996) and a modified physical map, based on the consensus map of Collins et al. (1996), adjusted to reflect data that have emerged from our FISH studies, are presented. In addition to YACs containing this set of genetic markers, we also isolated YAC clones containing a number of genes that were known to be located in this region but had not been

finely mapped. These were selected on the basis of their potential involvement in the etiology of cleft palate (Table I).

Statistical analysis of chromosomal deletions

By analysing all cases of single, contiguous, non-mosaic autosomal deletions stored in the Human Cytogenetics Database (HCDB) (Schinzel, 1994) we have identified three chromosomal regions (2q32, 4p16-13, 4q31-35) where monosomy is non-randomly associated with CPO. However, HCDB searches alone do not differentiate cleft palate in the context of CL(P) cases from those cases that have CPO. As these are etiologically distinct subgroups of orofacial clefting and would be expected to have different causative genetic loci, the precise significance of these three chromosomal loci must necessarily be unclear. For a better insight into the phenotypes associated with deletion of these regions, the original case reports of all cases were obtained and reviewed [a full reference list is available from the authors]. A statistical reanalysis was then performed as before but confining the analysis to confirmed CPO cases. Briefly, the distribution of deletions of regions including bands on chromosomes 4 or 2q in CPO patients was determined. The observed number of CPO-associated deletions of each band was compared with the expected number, calculated from the distribution of all band deletions on chromosomes 4 and 2q in HCDB. The number of deletions of any band was taken to follow a Poisson distribution, since this number is usually small. Confidence limits for the observed number of deletions and the significance of any deviation from expectation were calculated as described by Vasarhelyi and Friedman (1989).

RESULTS

Patient phenotypes

Initial clues to the existence of a CPO locus on 2q32-33 came through the identification of a patient (Case 2) with a de novo balanced reciprocal translocation

t(2;11)(q33;p14). We then identified a second patient (Case 1), again with CPO and a de novo translocation [t(2;7)(q33;p21)] involving the same cytogenetic band on chromosome 2. Both patients were examined and their clinical appearances are strikingly similar.

FISH Analysis

Initial chromosome painting studies confirmed that the sizes of the translocated 2q fragments were approximately the same in both patients (Fig. 1A, 1B). To attempt to establish whether the breakpoints in Cases 1 and 2 had occurred within the same region of chromosome 2q32, single locus FISH analysis was then conducted, using a large collection of YACs containing markers mapping within the 2q32-33 region. The results of this FISH study are summarised in Table 1. YACs containing the candidate genes FN1 (fibronectin) (Iamaroon 1996), IGFBP5 (Ferguson et al. 1992), IGFBP2 (not shown) and IHH (Indian Hedgehog) (Leek et al 1997, not shown) were all found to map distal (telomeric) to both chromosome 2 breakpoints.

Markers flanking both patients' 2q breakpoints were next identified by FISH (Fig. 3A, B). The signal generated by YACs containing D2S311 is present on both the normal and derivative copies of chromosome 2, in both Cases 1 and 2. Thus D2S311 is proximal (centromeric) to both breakpoints; this is clearly shown through simultaneous hybridization to the centromeric probes D2Z1 and either D7Z1 or D11Z1. In contrast, FISH with YACs containing D2S309 (though chimeric), or D2S116 gave signals lying distal to the breakpoint in both individuals. This suggested that both breakpoints lie within a common region of some 2.5 Mb of 2q32. To reduce this interval further, YACs 14HA2 (containing D2S2189), 26IF5 (containing D2S1384/D2S307/ CTLA4/D2S105/D2S72) and 11GG8 (containing D2S115) were analysed. All were found to map distal to the 2q32 breakpoint in both patients. In this way, by systematic FISH analysis, the breakpoint region in both these patients with cleft palate has been localized to an interval, which may be as small as 0.3 Mb, according to current maps (Collins et al. 1996).

On the basis of two independent lines of evidence, we suggest that a previously unrecognised locus causing cleft palate resides in chromosome region 2q32. Our suggestion relies on the integration of clinical, cytogenetic, molecular and statistical data. Our studies were initiated by the observation of two unrelated children with strikingly similar clinical features, each having a de novo cytogenetic rearrangement apparently involving the same band on chromosome 2q. Both girls have cleft palate. They also both have mild learning difficulties and a strikingly similar facial appearance. While their facial dysmorphisms are subtle, these and other clinical features are reminiscent of those seen in velocardiofacial syndrome (VCFS, OMIM 192430). It would appear, however, that these girls do not have VCFS, as neither of them has a cardiac malformation, nor the microdeletion of 22q11.22 seen in most cases of VCFS (Scrambler et al., 1992). The existence of non-22q-deleted phenocopies of VCFS is well recognized (Daw et al., 1996), so that it is possible that a 2q32 locus accounts for a proportion of such cases.

We isolated markers for this region of chromosome 2q, which allowed us to isolate a large number of genomic reagents for precise delineation of the 2q breakpoints in both cases. The 30 separate YACs that have been isolated provide good representation of this 2.5 Mb region. The average insert size in the YAC library used is 350 kb (Arand et al. 1990).

The initial G-banded cytogenetic studies performed on both patients indicated the existence of a common breakpoint at 2q33. However, our single locus FISH studies collectively strongly suggest that the breakpoint in both Case 1 and Case 2 lies in 2q32. This discrepancy may result from the known bias in reporting of breakpoints in favor of Giemsa-pale bands, or it may be that the true breakpoint is at the 2q32-2q33 band junction. More importantly, both breakpoints mapped to a very small chromosomal region between D2S311 and D2S309, strongly suggesting that the same single gene is disrupted in both patients. These flanking markers are estimated to map at 207.169 Mb and 207.462 Mb respectively, in the current version of the Location Database LDB (Collins et al. 1996; ttp://cedar.genetics.soton.ac.uk/public

html/). The cleft palate gene was thus localized by our studies to a region as small as 0.3 Mb. Two additional genetic markers (D2S374 and D2S1413) have been mapped into this interval. A genomic clone, YAC 854H8, crosses both patients' breakpoints and represents the cloning of this cleft palate gene.

The t(2;7)(q33;p21) and t(2;11)(q33;p14) chromosomes available in the two patients described here have proved useful for confirming the marker order in this region of 2q33, to some extent. The internal consistency of the separate FISH analyses with each patient and each marker is encouraging. The breakpoint acts as a useful anchor point for the genetic map in this region.

Independent support for the existence of an important cleft palate locus at 2q32 has been obtained from our statistical analysis [Brewer et al, 1998] showing that cytogenetically visible abnormalities associated with CPO are not randomly distributed in the genome, and that deletions involving chromosome 2q32 are particularly likely to result in CPO. Association and/or linkage studies using markers in this region, and a search for chromosomal microdeletions within 2q32 in cases of non-syndromic CPO provide further support for our hypothesis. However, ultimate proof that 2q32 contains a CPO-causative genetic locus has been provided by the cloning of a gene whose function is disrupted by both breakpoints in these patients and the demonstration of mutations within this gene in cytogenetically normal individuals with CPO.

REFERENCES

Beiraghi S, Foroud T, Diouhy S, Bixler D, Conneally PM, Delozierblanchet D and Hodes MS (1994). Clinical Genet., 46, 255-256.

Bitgood MJ, McMahon AP. (1995). Dev. Biol.;172:126-38.

Brewer C et al, (1998) Amer J. Hum. Genet., 63, 1153-1159.

Carter CO, Evans K, Coffey R, Roberts JAF, Buck A, Roberts MF (1982). J Med Genet. 19: 329-331.

Christensen K, Fogh-Andersen P. Cleft Palate-Craniofacial Journal 30:469-474.

Christensen K, Mitchell LE (1996). Am. J. Hum. Genet. 58: 182-190.

Collins A, Frezal J, Teague J Morton NE (1996). Nature Genet 13 458.

Dib C, Fauré S, Fizames C, Samson D, Drouot N, Vignal A, Millasseau P, Marc S, Hazan J, Seboun E, Lathrop M, Gyapay G, Morissette J and Weissenbach J (1996). Nature, 380, 152-154.

Dixon MJ and Ferguson MWJ (1992) Arch. Oral Biol., 37, 395-410.

Ferguson MWJ (1988). Development (Suppl.), 103, 41-60.

Ferguson MWJ (1994). Nature Genet., 6, 329-330.

Ferguson MWJ, Sharpe PM, Thomas BL and Beck F (1992). J. Anat., 181, 219-238.

FitzPatrick DR, Kondaiah P, Dehnex F and Akhurst R (1990) Development, 109, 585-595.

FitzPatrick DR, Farrall M (1993). J. Craniofac. Genet. Dev. Biol.;13, 230-235.

Fitzpatrick DR, Raine PAM, Boorman JG (1994). J. Med. Genet.;31,126-129.

Fogh-Andersen P (1942). Inheritance of harelip and cleft palate: contribution to the elucidation of the etiology of the congenital clefts of the face. Copenhagen,.

Gashler AL, Bonthron DT, Madden SL, Rauscher FJ, Collins T and Sukhatme VP (1992). Proc. Natl. Acad. Sci, USA, 89, 10984-10988.

Hill L, Murphy M, McDowall M and Paul AH (1988). Journal of Epidemiology and Community Health, 42, 1-7.

Hogan B, Beddington R, Constantini F and Lacy E (1994). Manipulating the mouse embryo; A laboratory manual. Cold Spring Harbor Press.

Gingrich JC, Boehrer DM, Garnes JA, Johnson W, Wong BS, Bergmann A, Eveleth GG, Langiois RG and Carrano AV (1996). Genomics, 32, 65-74.

Iamaroon A and Diewert VM (1996). J. Craniofacial Genet. Dev. Biol., 16, 48-51.

Kaartinen V, Voncken JW, Shuler C, Warburton D, Bu D, Heirkerkamp N and Groffen J (1995). Nature Genet., 11, 415-421.

Leek JP, Moynihan TP, Anwar R, Bonthron DT, Markham AF, Lench NJ (1997). Cytogenet Cell Genet, 76,187-8.

Lidral AC, Romitti PA, Basart AM, Doetschman T, Leysens NJ, DaackHirsh S, Semina EV, Johnson LR, Machida J, Burds A, Parnell TJ, Rubenstein JLR and Murray JC (1998). Amer. J. Hum. Genet., 63, 557-568.

Liu HC, Mele C, Catz D, Noyes N, Rosenwaks Z (1995). J Assist Reprod Genet, 12:78-87.

Lyon MF, in : Genetic Variants and Strains of the Laboratory Mouse (1996). Ed. MF Lyon, S Rastan and SDM Brown. Vol. 1, third edition, Oxford University Press, Oxford.

Markus T and Booth P (1995). British Medical Journal, 311, 765.

Morishita M, Shiba R, Chiyo HA, Furuyama JI, Fujita H and Atsumi Y (1983). J. Oral. Maxillofacial Surgery, 41, 601-605.

Murray JC (1995) Am. J. Hum. Genet., 57, 227-232.

O'Rahilly R and Muller F (1987). Developmental stages in human embryos. Washington: Carnegie Institute of Washington, 204-208.

Ohsaki Y, Nagata K, Kurisu K (1995). Acta Anat (Basel), 153, 161-7.

Proetzel G, Pawlowski SA, Wiles MV, Yin MY, Boivin GP, Howles PN and Ding JX, Ferguson MWJ, Doetschman T (1995). Nature Genet., 11, 409-414.

Resnick N, Collins T, Atkinson W, Bonthron DT, Dewey CF and Gimbrone MA (1993). Proc. Natl. Acad. Sci., USA, 90, 4951-4595.

De la Rosa EJ, Bondy CA, Hernandez-Sanchez C, Wu X, Zhou J, Lopez-Carranza A, Scavo LM, de Pablo F (1994). Eur J Neurosci, 6, 1801-1810.

Satokata I and Maas R (1994). Nature Genet., 6, 348-356.

Shiang R, Lidral AC, Ardinger HH, Buetow KH, Romitti PA, Munger RG, Murray JC (1993). Am. J. Hum. Genet. 53: 836-843.

Shields ED, Bixler D, Fogh-Andersen P (1981). Clin. Genet., 20: 13-24.

Shields ED, Bixler D, Fogh-Andersen P (1981). Cleft Palate J. 16: 1-6, 1979.

Spielman RS, McGinnis RE and Ewens WJ (1993). Amer. J. Hum. Genet., 52, 506-516.

The Treacher-Collins Syndrome Collaborative Group (1996). Nature Genet., 12, 130-136.

Werler MM, Lammer EJ, Rosenberg L and Mitchell AA (1991). American Journal of Epidemiology, 134, 691-698.

Williams A, Shaw WC and Devlin HB (1994). British Medical Journal, 309, 1552.

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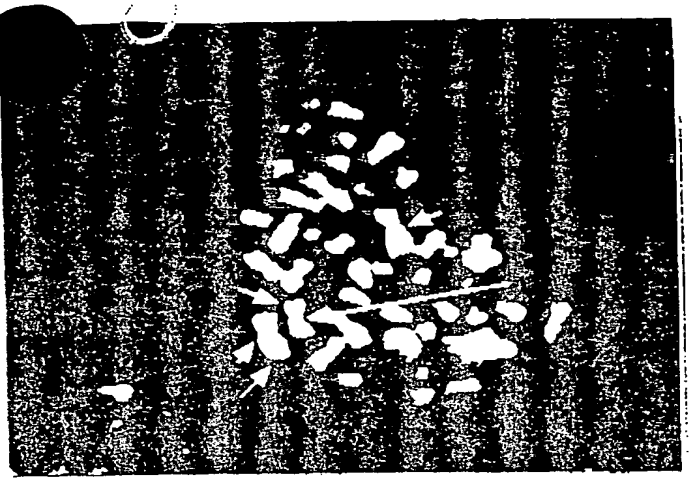

FIGURE 1A

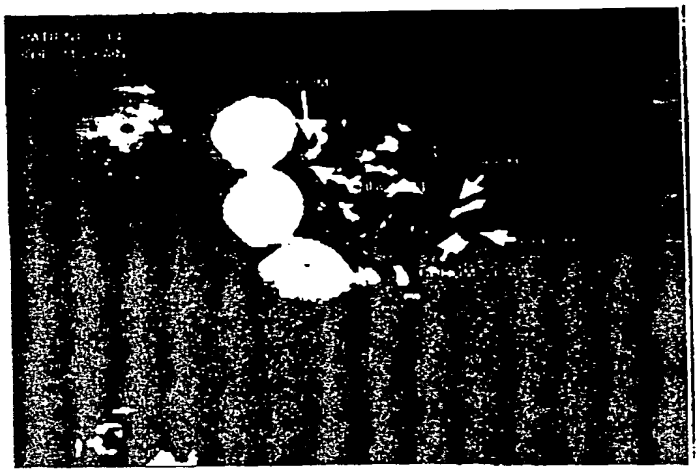

FIGURE 1B

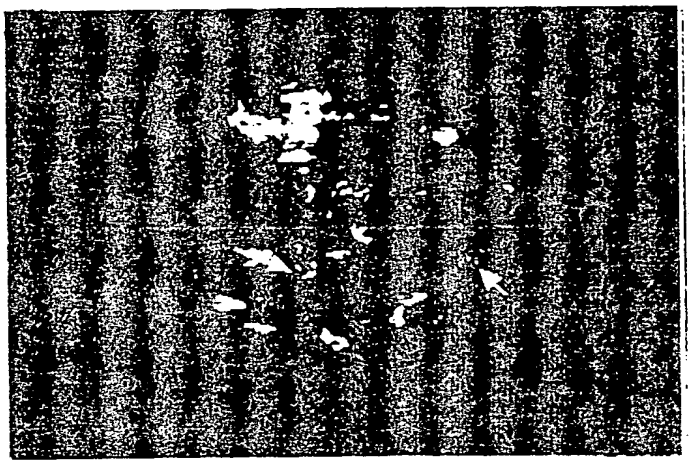

FIGURE 2A

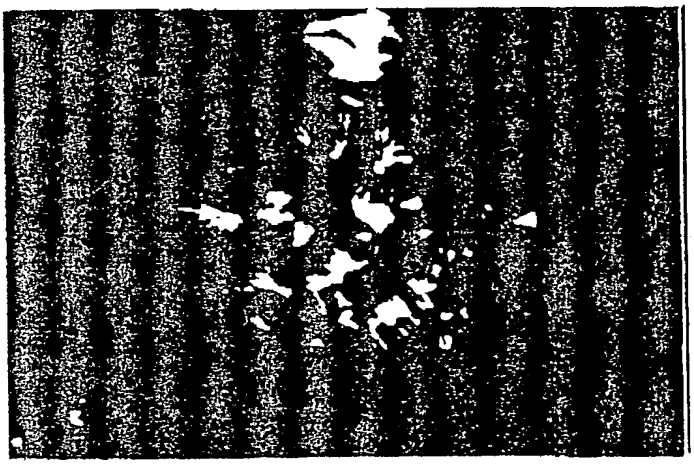

FIGURE 2B

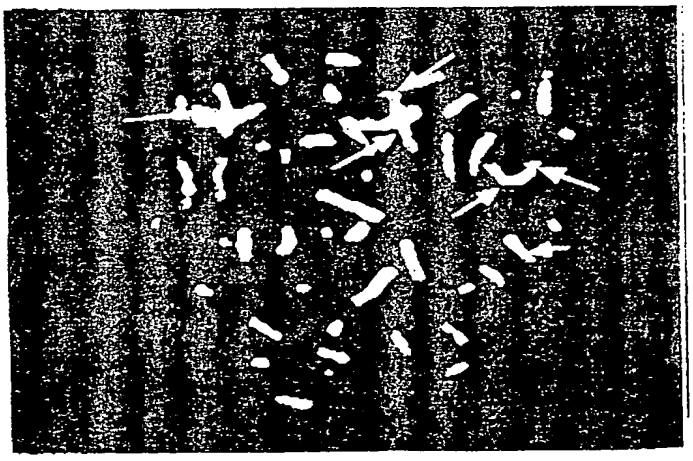

FIGURE 3A

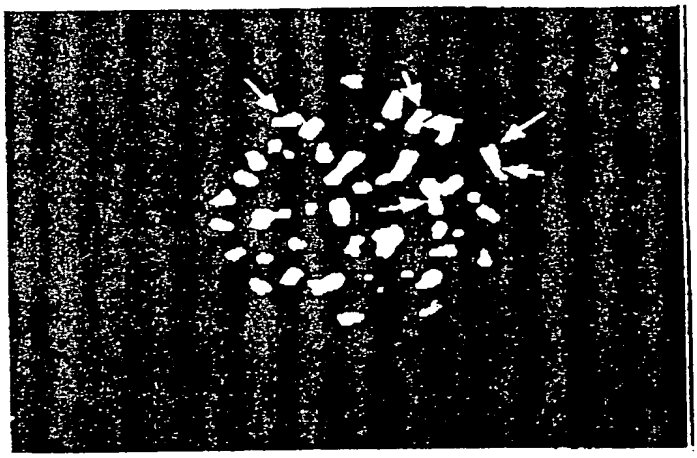

FIGURE 3B

FIGURE . 4.


PATIENT 0213
CEPH YAC 854H8
D7Z1
YAC
D7Z1
YAC
D2Z1
YAC
D2Z1
YAC

FIGURE. 5.

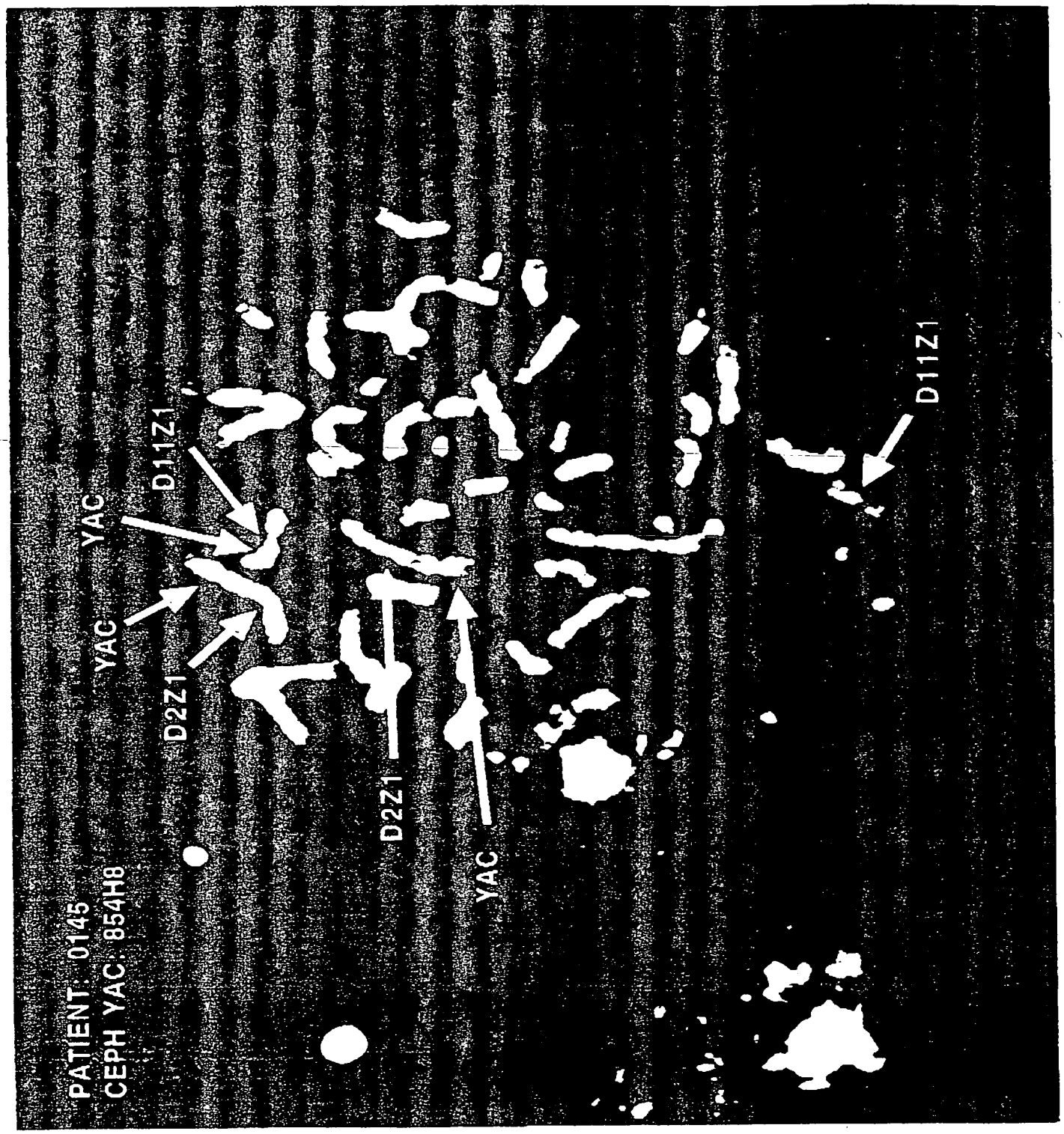
PATIENT: 0145
CEPH YAC: 854H8
YAC
YAC
YAC
D11Z1
D2Z1
D2Z1
D11Z1

TABLE 1: Oligonucleotides used in the Study.

Marker	Oligonucleotide Primers	YACs isolated	Chimeric by FISH	der(2) or der(7/11)
D2S311	dCAATTTTGAGCCCGGAAG dTGACTAGAAAGGCATTCCAGAG	17GD1 31CH5 33AC9	+ - -	
D2S115	dCAAGAACAGCCATATTGACTTGAAC dGGGTACAGCCCATGTGTGAG	11GG8	-	
D2S348	dAGGTGACCAGCAGCCTCT dGTAAAACGGACATATCCCCC	19ID10 21GA12 23EG11 32EB9	ND ND ND ND	
D2S72	dAGCTATAATTGCATCATTGCA dTGGTCTATAACGGTCTATG	26IF5	-	
D2S105	dCTCTACAGTTTATAACCAGC dTACACTGGATTCATATTCCC	26IF5	-	
CTLA4	dATTTCAATTTCCAAGAGCTGAGG dGCTGATGTGACAGAAACATCCC	26IF5 8IH5 13HC12 20AG2 22HB6	- ND ND ND ND	
D2S307	dCATGACCTGAAATAAACATAGACA dDAGCTTTTCCTGTAGGCTGTC	26IF5 22HB6 6BC7	- ND ND	
D2S1384	dAATAGAGGGCCCTTGCTTAA dTTTGGGATAAAAGGTATTTTGC	26IF5 22HB6 10GF2	- ND ND	
WI5293	dGAGTTAGACCCCGTCTAAAAAAA dACTCTCATCTCCTTCCTTGTTCC	26IF5 8BG7 14HA2 24GF8	- ND - ND	
D2S2189	dTACAAAAGGACTTGTCCAGGG dTCAAGATTGCCGTGAGGT	8BG7 14HAZ	ND -	

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		23CE7	ND
		24GF8	ND
D2S1271	dGGAAGGTCCAGATTAGAAG	15BA12	ND
	dAAGGGAAATAAAGAGAAGCAT	22HC8	ND
D2S116	dCAATCTCCACAAGTTGCTCA	6HA11	-
	dGGGATAGATAATTTAGGAGTGGG	13BE7	ND
		14DE4	ND
		16IB4	+
		28DE5	ND
D2S309	dGCTCTAGTAGGCTGGTTACATAA	4EC12	+
	dTTCCAAGAATAATGCAATCTCAG	31DH5	+
FN1	dTTGTTCCTACAGTATTGCGGG	7AH3	+
	dCCAACCCAAGATGCAAATG	11GH11	ND
		31GG9	-
		37HB8	ND
IGFBP5	dCTATTGGGGTTTCCCAGGAT	21EC3	-
	dTTTCCAATATTGGGGCATGT	22DB10	ND
IGFBP2	dCAGTAGACCGCAGCCAGC	7FA11	ND
	dGGAAAGCAAGAAGGAGCAGG	8HF12	ND
		21EC3	-
		22DB10	ND
IHH	dGGACTCCACCTGGAACTGC	35EF10	-
	dGAAAACTCGTAGTGAGAGCAG		

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TABLE 2: Genetic Map of CP-1 Region

A	Cen	B	Cen	
	D2S311		D2S311	der(2)
			D2S374	⇐
			D2S1413	⇐
	D2S2327		D2S115	der(7)/der(11)
			D2S348	der(7)/der(11)
	D2S2396		D2S72	der(7)/der(11)
			(D2S2327)	
	D2S374		(D2S2396)	
	D2S2217		D2S105	der(7)/der(11)
			CTLA4	der(7)/der(11)
	1.4cM		D2S307	der(7)/der(11)
			(D2S2217)	
	D2S2392		(D2S2392)	
	0.1		(D2S1740)	
	cM			
			(D2S2708)	
	D2S309		(D2S1837)	
			(D2S2684)	
	Tel		(D2S1367)	
			D2S1384	der(7)/der(11)
			WIS293	der(7)/der(11)
			D2S2189	der(7)/der(11)
			D2S1271	der(7)/der(11)
			D2S116	der(7)/der(11)
			D2S309	der(7)/der(11)
			Tel	

PCT/GB00/00003

14/1/2000

Harrison Goddard Foote